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SEROLOGICAL SCREENING TEST FOR ANY BOTULINUM
TOXIN TYPE

Final Report

by

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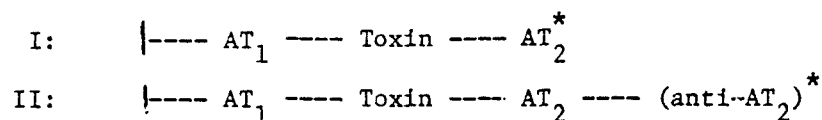
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INTRODUCTION

An enzyme-linked immunosorbent assay (ELISA) can theoretically be devised for any antigen. The three botulinum toxin types responsible for most human botulism cases have been assayed in separate ELISA tests whose detection limits are 50-100 mouse i.p. LD₅₀ of type A toxin (1), 400 LD₅₀ of type B toxin (2) and 80 LD₅₀ of type E toxin (3).

These botulinum toxin ELISA are based on the "double sandwich" principle but differ in how the enzyme is introduced into the test. The difference is illustrated in the following reaction sequences of the two methods:



where first antitoxin (AT₁) is adsorbed to inner wall of polystyrene tubes, second antitoxin (AT₂) is from an animal species different from that used to prepare AT₁, asterisk indicates enzyme-labeled reagent and anti-AT₂ is antibody for IgG of AT₂ but not of AT₁.

The double sandwich approach is possible because of multiple antigenic determinants of the toxin molecule. Immobilized AT₁ binds toxin by reacting with these determinants but some remain available to AT₂, either because they have not participated in the first antigen-antibody reaction and/or because AT₂ displaces AT₁. In any case, enough AT₁ remains bound to toxin so that the latter is specifically retained in the test system. In both procedures, the amount of AT₂ retained depends on the toxin concentration in the test toxin sample. In method I, AT₂ is measured directly from the enzymatic activity remaining after washing out unattached AT₂^{*}; in method II, retained AT₂ is measured indirectly by using its antigenic property to fix

enzyme carried by its antibody. Toxin amount in the sample is determined from a standard plot relating amounts of enzyme retained by known amounts of homologous type toxin.

The ELISA methods used antitoxins from a horse and a rabbit. Since they were raised with impure antigens, the equine antisera would react with other botulinum antigens in addition to toxin. Specificity for toxin was obtained by using rabbit antitoxins raised with immunogens made of purified neurotoxins (mol wt about 150,000). ELISA for B and E toxins used IgG fraction from antitoxin prepared in rabbit as AT₁ and horse antitoxin as AT₂ while type A toxin ELISA used horse antitoxin as AT₁ and rabbit antitoxin as AT₂.

Thus, ~~methods~~ methods are available to test samples directly for A, B or E botulinum toxin, if the toxin type is known. If the type is unknown, testing is complicated in that separate tests for the three types become necessary. This need for multiple tests on one specimen is a distinct disadvantage when many samples are to be examined.

The present work was concerned with adapting one of the published ELISA methods into a screening procedure which would give a positive reaction regardless of the toxin type in the sample. Method II was tried by substituting mixtures of antitoxins for the single antitoxin of the type specific ELISA. The advantage of the method over I was the need for only one enzyme-labeled reagent since (anti-AT₂)^{*} reacts with rabbit IgG of different antitoxic specificities.

MATERIALS AND METHODS

PURIFIED NEUROTOXINS.

Type A. Neurotoxin in twice recrystallized type A toxic complexes was

isolated by affinity chromatography (4).

Type B. The Okra strain culture strain was grown in a medium consisting of 1% N-Z Amine type B and 1% N-Z Case (both from Humko Sheffield), 1% yeast extract, 1% glucose, 0.2% calcium lactate and 0.5% sodium thioglycollate, adjusted to pH 7.6 before autoclaving. Titters of 1×10^6 mouse i.p. LD₅₀/ml were obtained when cell lysis occurred on about fourth day of incubation at 35 C.

The whole culture was made to pH 3.8 with concentrated H₂SO₄ and was then allowed to stand overnight. The precipitate was collected by siphoning off the supernatant fluid. The harvested sediment was washed with water and then extracted with 0.1 M Tris-HCl buffer, pH 7.5, at rate of 250 ml for precipitate from 12 l of culture, for 30 min at room temperature. Undissolved material was sedimented by centrifugation and the pellet re-extracted with fresh buffer of same volume. Most of the recoverable toxin was in these two extracts; if not, the extraction procedure was repeated.

The pool of the two extracts with highest toxicities was made 58% saturated with (NH₄)₂SO₄ by dissolving dry salt. The resulting precipitate was collected by centrifugation and was dissolved in the Tris-HCl buffer at rate of 100 ml buffer for precipitate from 12 l of culture. The extract was clarified by centrifugation and dialyzed for 2 days at 4 C against several changes of 0.05 M acetic-acetate buffer, pH 4.5.

The precipitate formed during dialysis was collected by centrifugation and was extracted twice with 50 ml volumes of the acetate buffer containing 0.5 M NaCl. The extract was equilibrated to 0.05 M citric-citrate buffer, pH 5.5 by dialysis and was chromatographed on DEAE-Sephadex A-50 (5). Fractions of first unretarded protein peak having A₂₆₀/A₂₇₈ of 0.5 to 0.55 were pooled and its protein precipitated at 55% saturation of (NH₄)₂SO₄. The

resulting precipitate was dissolved in 0.15 M Tris-HCl buffer, pH 7.4 and was chromatographed on DEAE-Sephadex A-50 equilibrated with same buffer.

Highly purified type B neurotoxin was usually obtained in the first protein peak eluted by a NaCl gradient up to 0.15 M in the Tris-HCl buffer. In some cases, a repeat chromatography was needed to obtain a preparation of acceptable purity.

Type E. This neurotoxin was purified by a published procedure (6).

Tests for purity. Samples containing at least 50 ug protein were electrophoresed in SDS-polyacrylamide gels (7). When sample was pure, untrypsinized type A and B and trypsinized type E preparation each showed a single protein band representing units of about 150,000 daltons. When treated for reduction of disulfide bonds, these units were replaced by molecules of about 100,000 daltons (H chain) and about 50,000 daltons (L chain). Contaminants, when present, were less than 1% of protein.

Purified neurotoxin preparations were reacted in gel immunodiffusion tests against homologous type antitoxin obtained by injecting a rabbit with toxoid made of a relatively crude toxin sample. All neurotoxins used as pure gave only a single immune precipitate line when different antigen concentrations were tested against several dilutions of antitoxin.

TOXICITY ASSAY.

When information of approximate toxicity was sufficient, as in following recovery by purification steps, the i.v. assay (8) was used. For more precise titrations, the i.v. method was used to determine the successive twofold serial dilutions which given i.p. would kill 0% to 100% of 4 mice. These dilutions were injected i.p. in 0.5 ml volumes and i.p. LD₅₀ obtained by the Reed and Muench method (9) by using deaths within 4 days of challenge.

ANTITOXINS.

AT₁. Equine antitoxins were obtained from the Chiba Serum Institute, Japan. These sera are responses to injections of purified "progenitor toxin" (toxic complex).

AT₂. A New Zealand, albino rabbit was immunized with one of the purified neurotoxin preparation. First dose was the intramuscular injection of 1.0 mg of toxoid (Formalin-treated toxin) homogenized in an equal volume of Complete Freund Adjuvant (Difco). One month later, the rabbit was given 100 ug of active toxin intracutaneously on each of three successive days. A similar injection series was given 10 days after the last dose of the booster series. The rabbit was bled out by cardiac puncture 8 to 10 days later.

Antisera were examined by gel immunodiffusion tests against several concentrations of crude, homologous type of toxin. None of the antisera had significant levels of antibodies to other than neurotoxin since only one immune precipitate line developed.

IgG fractions and titrations. The ELISA procedures used IgG fractions of antitoxins. IgG was preferentially precipitated from rabbit antisera at 35% and from horse antisera at 30% saturation with $(\text{NH}_4)_2\text{SO}_4$ (10). Further purification was obtained by chromatographing dissolved precipitate through Sephadex G-200 (11).

Antitoxic potency of IgG fraction was estimated by titrations using freshly prepared culture fluid as challenge toxin. Types A and B toxins were used directly; type E toxin was activated with trypsin before use. Twofold serial dilutions of IgG solution were each mixed with toxin so that 0.5 ml of the mixtures originally had 1,000 mouse i.p. LD₅₀. After

holding 10 min at 30 C, 0.5 ml of one mixture was injected i.p. into each of 4 mice. The greatest antitoxin amount leaving one LD₅₀ in the challenge volume was determined by the Reed and Muench method (9). The calculated amount of type E antitoxin was considered to contain one "international unit"; one unit of A or B antitoxin was assumed to be in 10 times the largest amount that left one LD₅₀. The different LD₅₀ neutralized by one unit of type E vs A or B antitoxins was based on one international standard unit of A or B antitoxin neutralizing about 10,000 LD₅₀ as compared to 1,000 LD₅₀ by a standard unit of type E antitoxin.

(Anti-AT₂)^{*}. This reagent was commercially available (Cappel Laboratory) goat anti-rabbit IgG-horse radish peroxidase conjugate. Several lots of sheep anti-rabbit IgG-horse radish peroxidase prepared in our laboratory by a published procedure (1) were satisfactory, but most tests were done with the commercial product in order that the time involved in preparation could be used for more productive work.

ENZYMATIC REACTION.

Substrate. ABTS (2,2'-azino-di-3-ethyl-benzthiozoline-6-sulfonate) was used as substrate for horse radish peroxidase (12). Working substrate solution was made freshly from stock solutions: 12.5 ml of citrate buffer (9.6 g citric acid in water adjusted to pH 4.0 with 1 N NaOH and made to 1 liter with H₂O), 12.5 ul of ABTS (0.545 g in 25 ml water), 25 ul of 3% H₂O₂ and diluting mixture with equal volume of water. Enzymatic reaction was stopped with freshly prepared solution made of 9.5 mg Na₄EDTA in 25 ml NaF solution (3.47 ml of 28.8 M HF, 6.0 ml of 1.0 N NaOH and water to 1 liter).

General procedure for ELISA. Capped polystyrene tubes of 13 x 100 mm (Falcon) were used. Toxin samples were cell-free culture fluids, with type

E toxin being trypsinized immediately before use. Incubations were done in a 30 C water bath with gentle shaking.

(1) Tubes containing 1.0 ml of IgG from horse antitoxin (AT_1) in 0.1 M carbonate buffer, pH 9.5 were incubated overnight.

(2) Fluid was removed by siphoning and tubes washed twice with water before adding 1.0 ml of 2.0% bovine serum albumin (fraction V; Sigma) in 0.1 M phosphate buffer-0.5 M NaCl, pH 7.2 (PBS).

(3) After 60 min incubation, fluid was removed and replaced with 1.0 ml of toxin diluted with BS.

(4) Tubes were incubated 60 min, emptied, washed twice with 1.5 ml of PBS and 1.0 ml of IgG of rabbit antitoxin (AT_2) in PBS added.

(5) After 60 min incubation, fluid was removed, tubes were washed with PBS and 1.0 ml of ABT substrate added.

(6) After 10 min incubation, enzymatic reaction was stopped by adding 1.0 ml of NaF-EDTA.

(7) Color was read at 414 nm. Background color, determined with tubes in which 2.5% serum albumin substituted for toxin, was subtracted. Results are averages of net optical density (O.D.) found in duplicate or triplicate tubes.

RESULTS

PRELIMINARY TITRATIONS.

Amounts of reagents to be used in ELISA monospecific for types A, B or E botulinum toxins were determined by titrations. The rationale is illustrated with the tests used to select the concentrations of reagents that would give an acceptable ELISA for type A toxin.

In the first titration, ELISA responses to different toxin amounts were determined with test systems using one of three possible AT_1 concentrations but the same AT_2 amount that was expected to be in slight excess of optimum. Plots of dose responses given by tubes using same AT_1 concentration gave three standard curves (Fig. 1). In this example using 2 units of AT_2 , the standard curve given by 1 unit of AT_1 was questionably better than that given by 2 units but superior to the one given by 4 units. The results indicated that incubating 1.0 ml of AT_1 containing 1.0 unit of antitoxic activity in polystyrene tubes results in tubes adsorbing sufficient anti-toxin so that they give dose related responses to toxin ranging from 10^2 to 10^5 LD₅₀/ml.

The second titration determined the amount of AT_2 to be used. Different units of AT_2 were tried in tubes treated with AT_1 concentration (1 unit) determined to be optimum by the first titration. The data (Fig. 2) indicated that 1 unit of AT_2 should give the best ELISA for type A toxin when optimum AT_1 is one unit.

The third titration determined the optimum concentration of enzyme-labelled anti- AT_2 for this combination of AT_1 and AT_2 . Of the three possibilities tested (Fig. 3), the 1:2,000 dilution gave greatest color change but the high color of its blank resulted in net O.D. slightly lower than the 1:4,000 dilution which was chosen to be the best.

Similar titrations indicated that an acceptable ELISA for type B toxin would be obtained by using both AT_1 and AT_2 at 1.0 unit per ml and conjugate at 1:4,000. The amounts determined for ELISA of type E toxin were 5 units in AT_1 , 2 units in AT_2 and 1:5,000 dilution of (anti- AT_2)^{*}. Since it gave results almost as good as the 1:5,000 dilution and was best in the types A and B ELISA, a 1:4,000 dilution was chosen.

ELISA STANDARD CURVES.

Optimum units of AT_1 and AT_2 for the ELISA differed somewhat with the lots that were prepared from same stock antitoxins. Optimum combinations for type A and B toxin tests ranged between 2 units of AT_1 with 1 unit of AT_2 to 1 unit of AT_1 with 2 units of AT_2 . One likely reason for the range is that the assay of antitoxic units is less rigorous than the formal method using standard antitoxin preparations. Thus, although they are reported to have contained different units, solutions of AT_1 (or AT_2) used at different times as having optimum concentration may have had same unitage.

Standard curves of a toxin type determined at different times with different lots of AT_1 and AT_2 were reasonably comparable. When the lack of precision of ELISA results is considered, those of type A and B toxins were not greatly different from each other, but that of type E was distinct from these two in that the toxin retained more enzyme than an equivalent LD_{50} of A or B toxin (Fig. 4).

SCREENING ELISA.

The screening method was based on using AT_1 and AT_2 that would react with any one of the three possible toxin types. The three type specific AT_1 were combined so that 1.0 ml of the mixture had each at the concentration optimum for coating tubes of the type specific ELISA. The pool of the particular reagent lots used to obtain the data to be reported had 1 unit of anti-A, 1 unit of anti-B and 4 units of anti-E activities. AT_2 was a mixture also; the three type specific rabbit IgG were pooled in 1.0 ml volume on a similar basis (1 unit of anti-A, 2 of anti-B and 3 of anti-E). $(Anti-AT_2)^*$ was 1.0 ml of an 1:4,000 dilution of a commercial preparation.

The screening ELISA gave a positive reaction for botulinum when samples

had any one of the three toxin types. Standard curves made from responses to different doses of the same toxin type were comparable to that obtained in the type specific ELISA of the homologous type toxin (Fig. 5 vs 4). Thus, standards of the three toxins obtained in the screening ELISA had relationships similar to those of the type specific method: those of type A and B were, within the precision limits, very similar but differed from that of type E toxin. The data indicated that the screening ELISA should quantitate LD_{50} as well as the type specific method in instances where only one toxin type is present and its type is known so that the correct standard curve will be used for the quantitation.

Samples having different combinations of type A, B and E toxins were tested in the screening method. Since standards of different trials varied to some extent, further study is needed to more firmly quantitate the amounts of enzyme retained by mixtures of toxins of various total LD_{50} . However, available data (Table 1) suggested that total LD_{50} in mixtures of A and B toxins can be estimated by using the standard curve of either toxin type. This was possible because the standards of these toxin types are similar and because such mixtures react as if they ^{have} only one toxin type at a concentration equal to the sum of LD_{50} of the A and B components.

Estimates of total LD_{50} was not possible with mixtures of type E and A or B toxin types. In such cases, quantity of specifically retained enzyme approximated the total retained when the LD_{50} of the individual toxin types are tested separately. With this kind of relationship, none of the available standard curves was suitable for determining total LD_{50} .

DISCUSSION

When mixtures of anti-A, anti-B and anti-E IgG are substituted, the toxin

type specific ELISA becomes a screening method which gives a positive reaction for botulinum toxin regardless of which toxin type is in the sample. However, if the type responsible for the reaction is to be determined, the sample must be retested in the individual type specific ELISA. If these follow-up tests show only one toxin type, its LD_{50} can be estimated from the data of the screening ELISA. Total LD_{50} can be similarly determined if the sample is a mixture of type A and B toxin types, but not when it is a mixture of type E and A (or B) toxins.

It should be possible to avoid the limitations of the tube-screening ELISA by substituting nylon beads for polystyrene tubes as carrier of AT_1 . Preliminary work with type A toxin assay shows that anti-A AT_1 can be covalently bound to one bead (13) in a quantity sufficient to give dose responses with up to 10^4 LD_{50} of the toxin. Thus, it should be possible to prepare other beads, which are marked for identification purposes, carrying similar amounts of anti-B and anti-E AT_1 , respectively. One bead of each specificity is then placed in a common vessel and reacted with the sample. The beads are then treated with AT_2 containing anti-A, -B and -E IgG and then with (anti- AT_2)^{*}. The beads are now separated into individual tubes and reacted with substrate of the enzyme. Since the toxin type specificities of the beads are known, the one(s) which has specifically retained enzyme indicates the toxin type(s) in the sample. LD_{50} of each toxin type can also be obtained by using standard curves of the homologous toxin(s). It can be suggested that this approach might be useful when there is need to screen samples for several completely unrelated antigens.

The AT_1 and AT_2 were used in the type specific ELISA in amounts such that the one of lower concentration, if there is a real difference, neutralizes

about 10,000 LD₅₀ of type A or B toxin and about 2,000 LD₅₀ of type E toxin. If the lower concentration was used as AT₁, neutralizing capacity was even lower since polystyrene tubes do not adsorb all IgG in the solution. In spite of this theoretical restriction to the upper limit of the standard curves, dose-response relationships of A and B toxins reached 100,000 LD₅₀ and that of type E reached 10,000 LD₅₀. The limit of standard curves for A and B toxins in the screening ELISA were also about 100,000 LD₅₀.

The difference between theory and experiment is also seen in published ELISA for botulinum toxin (1, 2, 3). Although inability of ELISA to discriminate between toxic antigens and those which have lost this biological activity (14) could contribute to the discrepancy, it is probably not of major importance. A more likely explanation is the multiple antigenic determinants of the toxin molecule. H and L chains are different antigens and the former probably has at least two different determinants (15). If antibodies to these several determinants are not equally potent in neutralizing toxicity, units based on mouse protection tests may not be the best criterion to use in determining optimum amounts of AT₁ and AT₂.

Part of the difficulty could be avoided and the detection limit of ELISA possibly lowered if AT₁ is antibody specific for L (or H) chain and AT₂ is antibody specific for H (or L) chain. Monoclonal antibodies for determinants of L and H chain could be substituted. If such chain specific antibodies are used in the ELISA, toxin would be specifically retained via only one chain; all determinants of the other chain will be available to react in the steps by which retained toxin is quantitated.

REFERENCES

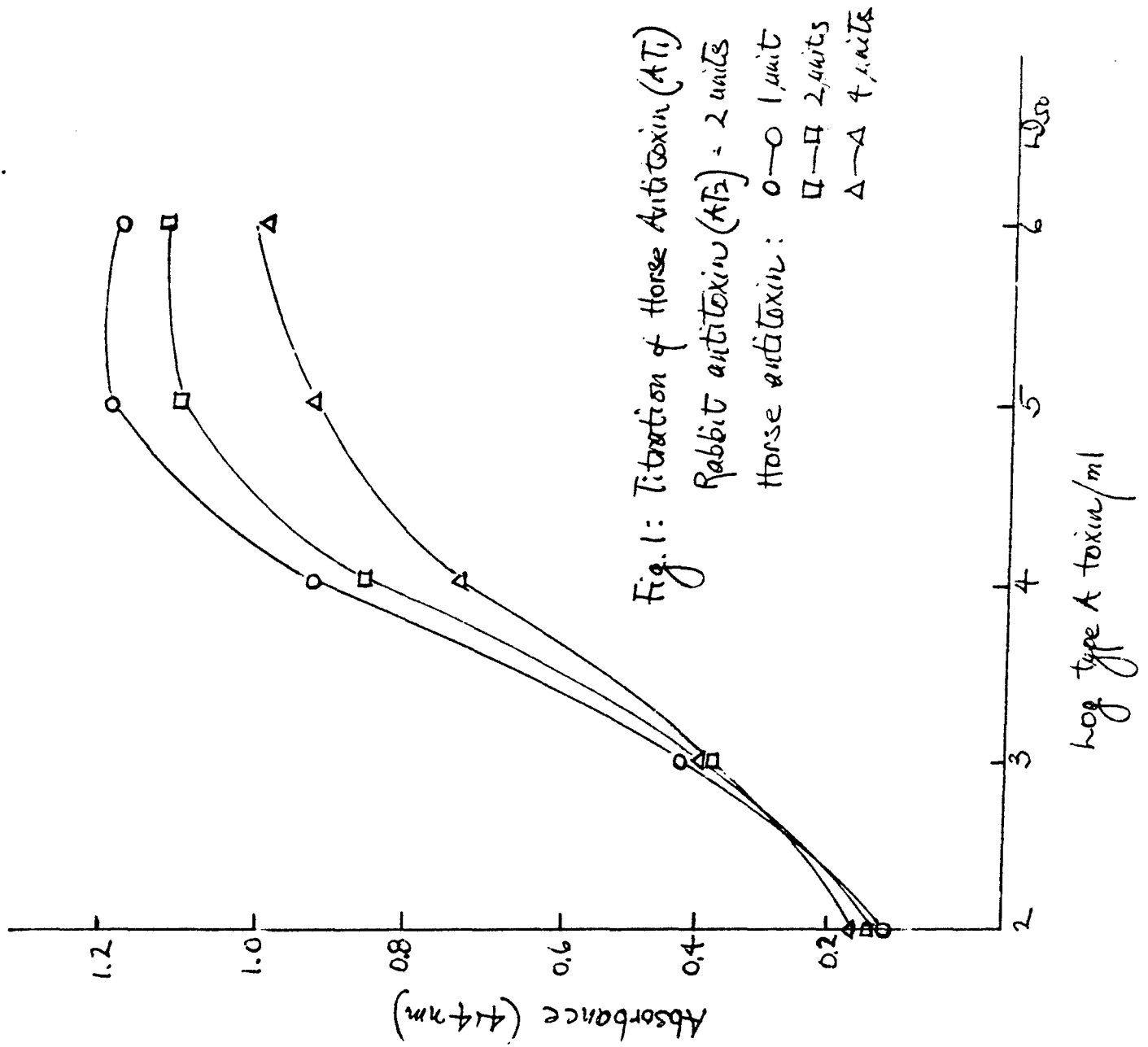
1. Notermans, S., J. Dufrenne, and M. van Schothorst. 1978. Enzyme-linked immunosorbent assay for detection of Clostridium botulinum toxin type A. Jap. J. Med. Sci. Biol. 31:81-85.
2. Kozaki, S., J. Dufrenne, A. M. Hagenars, and S. Notermans. 1979. Enzyme-linked immunosorbent assay (ELISA) for detection of Clostridium botulinum type B toxin. Jap. J. Med. Sci. Biol. 32:199-205.
3. Notermans, S., J. Dufrenne, and S. Kozaki. 1979. Enzyme-linked immunosorbent assay for detection of Clostridium botulinum type E toxin. Appl. Environ. Microbiol. 37:1173-1175.
4. Moberg, L. J., and H. Sugiyama. 1978. Affinity chromatography purification of type A botulinum neurotoxin from crystalline toxic complex. Appl. Environ. Microbiol. 35:878-880.
5. Sugiyama, H., L. J. Moberg, and S.L. Messer. 1977. Improved procedure for crystallization of Clostridium botulinum type A toxic complexes. Appl. Environ. Microbiol. 33:963-966.
6. Kitamura, M., S. Sakaguchi, and G. Sakaguchi. 1969. Significance of 12S toxin of Clostridium botulinum type E. J. Bacteriol. 98:1173-1178.
7. DasGupta, B. R., and H. Sugiyama. 1972. A common subunit structure of Clostridium botulinum type A, B and E toxins. Biochem. Biophys. Res. Comm. 48:108-112.
8. Boroff, D. A., and U. Fleck. 1966. Statistical analysis of a rapid in vivo method for titration of the toxin of Clostridium botulinum. J. Bacteriol. 92:1580-1581.
9. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27:493-497.
10. Hebert, G. A., P. L. Pelham, and B. Pittman. 1973. Determination of the optimum ammonium sulfate concentration for the fractionation of rabbit, sheep, horse, and goat antisera. Appl. Microbiol. 25:26-36.
11. Hudson, L., and F. C. Hay. 1976. Practical immunology. Blackwell Scientific Publication, Oxford.

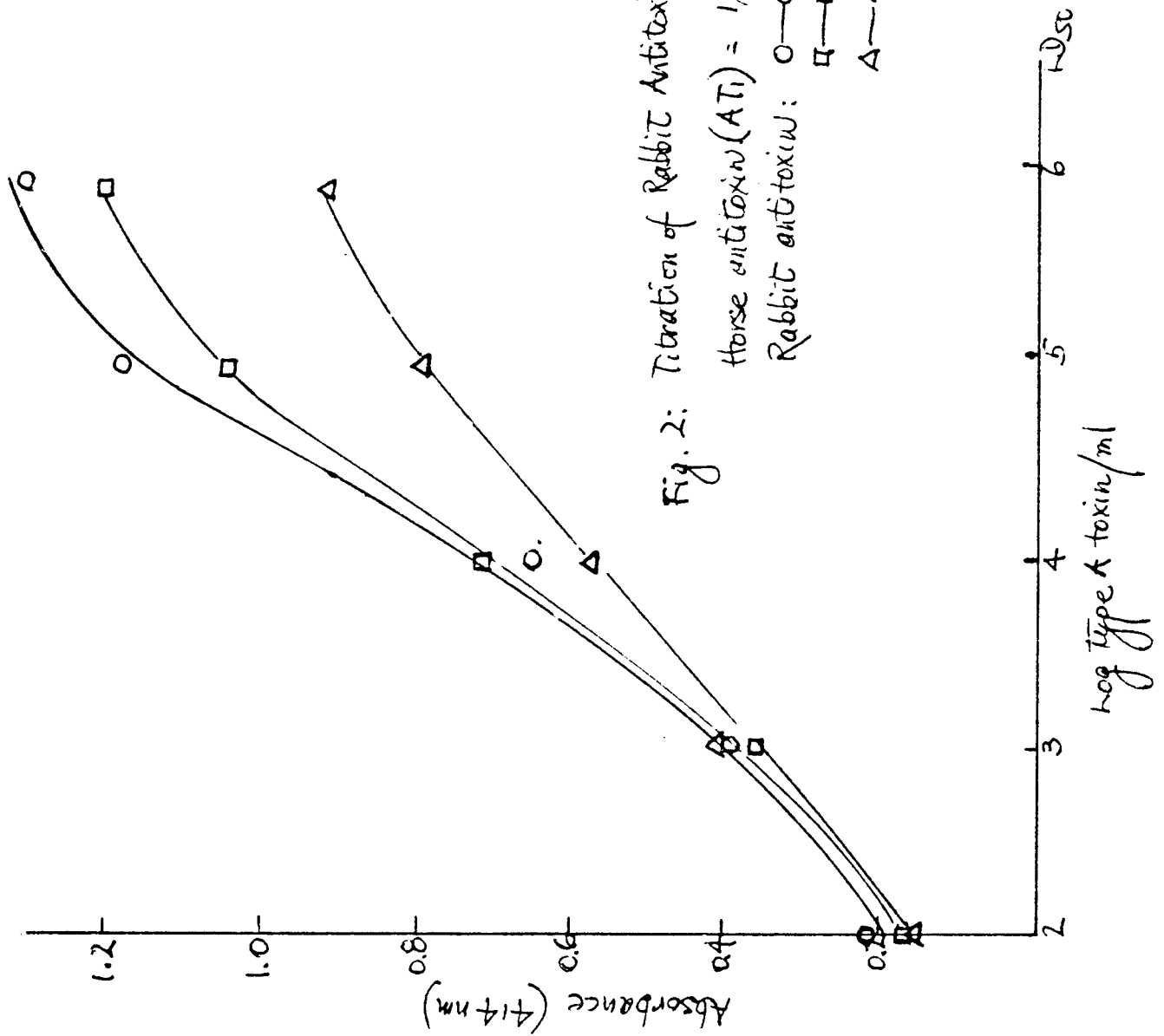
12. Pestka, J. J., P. K. Gaur, and F. S. Chu. 1980. Quantitation of aflatoxin B₁ and aflatoxin B₂ antibody by an enzyme-linked immunosorbent microassay. Appl. Environ. Microbiol. 40:1027-1031.
13. Hendry, R.M., and J. E. Hermann. 1980. Immobilization of antibodies on nylon for use in enzyme-linked immunoassay. J. Immunol. Meth. 35:285-293.
14. Betley, M. J., and H. Sugiyama. 1979. Noncorrelation between mouse toxicity and serologically assayed toxin Clostridium botulinum type A culture fluids. Appl. Environ. Microbiol. 38:297-300.
15. Sugiyama, H. 1980. Clostridium botulinum neurotoxin. Microbiol. Rev. 44:419-448.

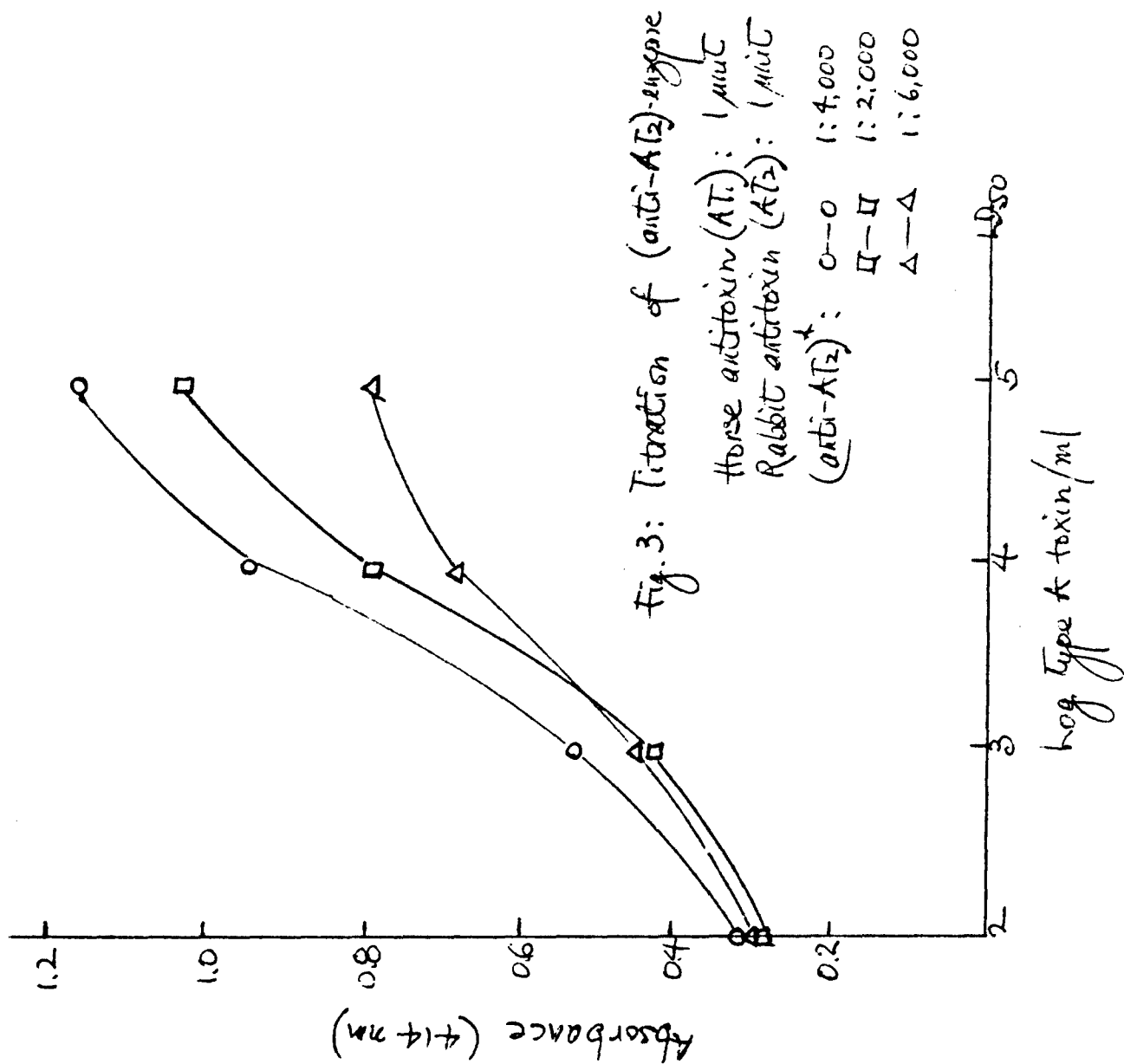
Table 1. Responses (color at 414 nm) of screening ELISA tested with different combinations of type A, B and E toxins.

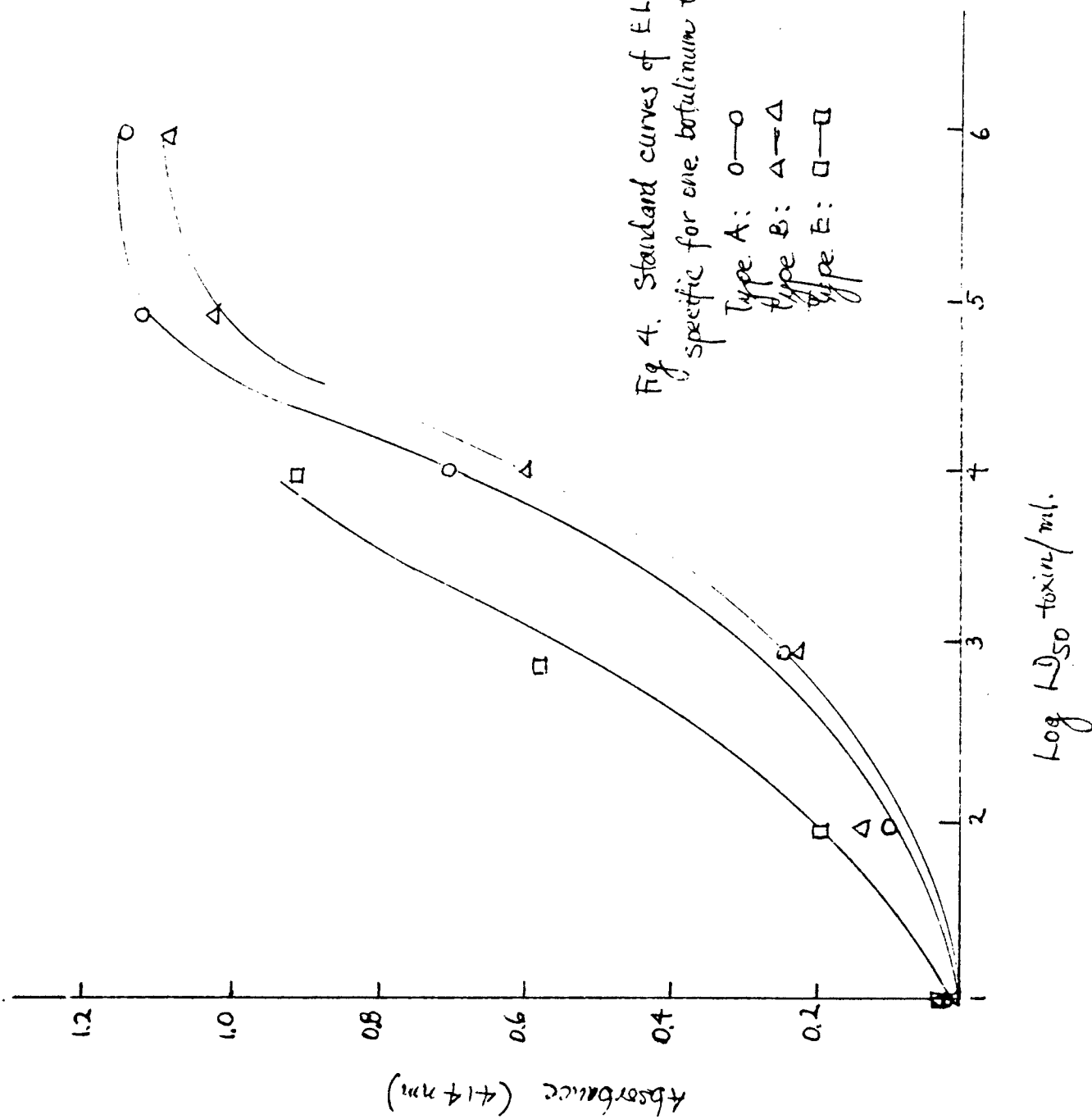
Log ₁₀ of toxin			Optical Density
A	B	E ¹	
4	4	-	0.70
2	4	-	0.55
4	2	-	0.60
2	2	-	0.12
4	-	3	1.17
4	-	2	0.78
2	-	2	0.36
-	4	2	0.70
2	2	2	0.39

¹Trypsinized.









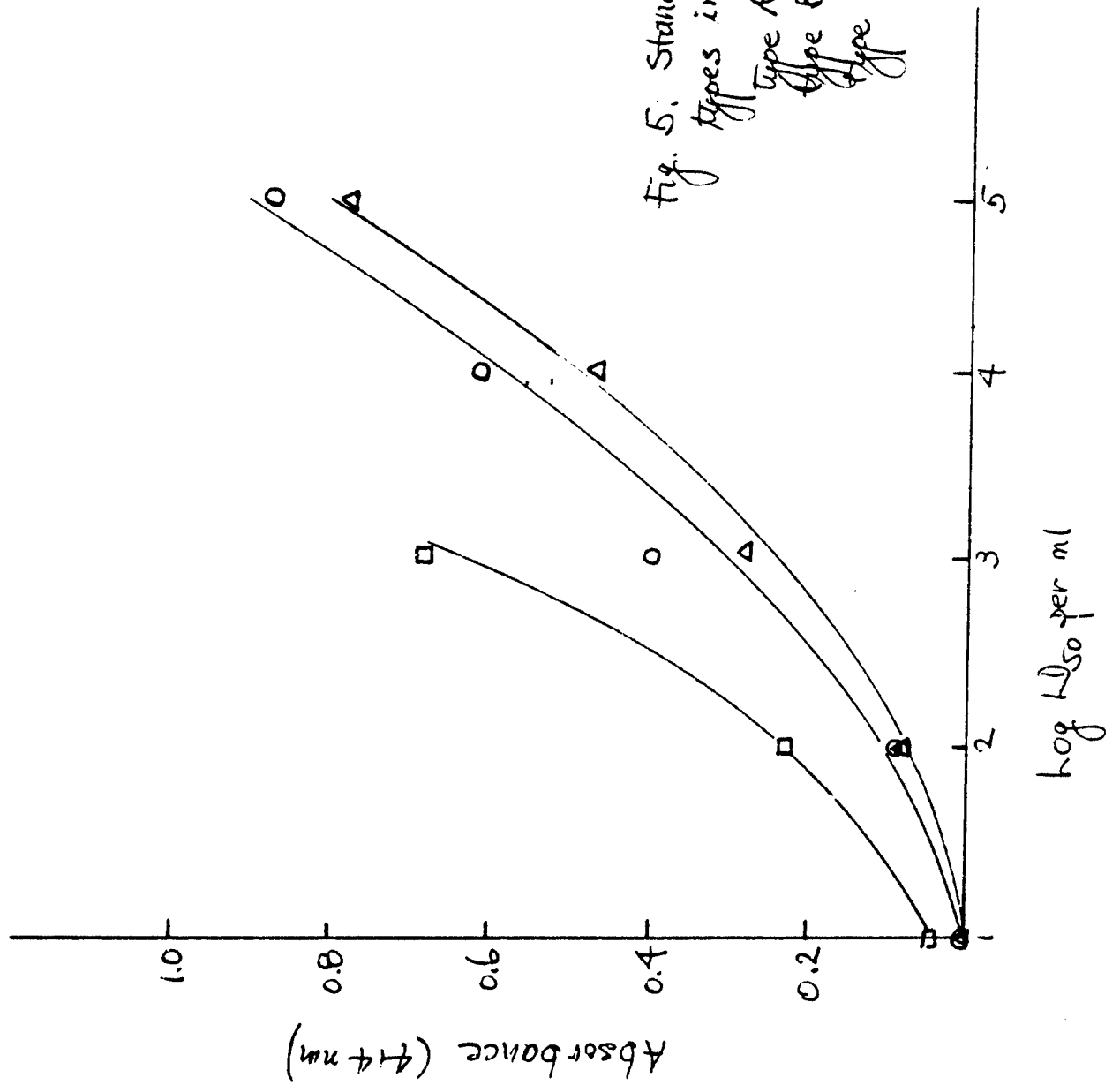


Fig. 5. Standard plots for toxin types in screening ELISA

Type A: ○—○

Type B: △—△

Type E: □—□